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Cell-Surface Expression of *Aspergillus saitoi*-Derived Functional α-1,2-Mannosidase on *Yarrowia lipolytica* for Glycan Remodeling

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Expression of proteins on the surface of yeast has a wide range of applications, such as development of live vaccines, screening of antibody libraries, and use as whole-cell biocatalysts. The hemiascomycetes yeast *Yarrowia lipolytica* has been raised as a potential host for heterologous expression of recombinant proteins. In this study, we report the expression of *Aspergillus saitoi* α-1,2-mannosidase, encoded by the *msdS* gene, on the cell surface of *Y. lipolytica*. As the first step to achieve the secretory expression of msdS protein, four different signal sequences-derived from the endogenous *Y. lipolytica* Lip2 and Xpr2 prepro regions and the heterologous *A. niger* α-amylase and rice α-amylase signal sequences-were analyzed for their secretion efficiency. It was shown that the YlLip2 prepro sequence was most efficient in directing the secretory expression of msdS in fully *N*-glycosylated forms. The surface display of msdS was subsequently directed by fusing GPI anchoring motifs derived from *Y. lipolytica* cell wall proteins, YlCwp1p and YlYwp1p, respectively, to the C-terminus of the Lip2 prepro-msdS protein. The expression of actively functional msdS protein on the cell surface was confirmed by western blot, flow cytometry analysis, along with the α-1,2-mannosidase activity assay using intact *Y. lipolytica* cells as the enzyme source. Furthermore, the glycoengineered *Y. lipolytica* Δ*och1*Δ*mpo1* strains displaying α-1,2-mannosidase were able to convert $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ efficiently on their cell-wall mannoproteins, demonstrating its potential used for glycoengineering *in vitro* or *in vivo*.

Keywords: *Yarrowia lipolytica*, surface display, a-1,2-mannosidase, GPI-anchor

Introduction

Expression of proteins on the cell surface has wide ranging applications in molecular biology and industrial biotechnology, such as the development of live vaccines (Lee *et al.*, 2000; Abe *et al.*, 2003), construction and screening of a displayed peptide/antibody library (Boder and Wittrup, 1997), bioadsorbents for the removal of harmful heavy metals (Xu and Lee, 1999; Bae *et al.*, 2000), and whole-cell biocatalysts (Richins *et al.*, 1997). Surface display expression of heterologous proteins on microbial cells is generally carried out using anchoring motifs of cell wall proteins (Ueda and Tanaka, 2000). The traditional yeast *Saccharomyces cerevisiae* has been extensively used as host for yeast surface display, because *S. cerevisiae* is a GRAS (generally recognized as safe) organism with a rigid cell wall, that makes this yeast well-suited for several applications related to the healthcare sector. Unlike *S. cerevisiae*, very little information is available on the display of heterologous proteins on the surface of other yeast species, although attention to other non-conventional yeasts including *Kluyveromyces lactis*, *Hansenula polymorpha*, and *Pichia pastoris* as host for surface display has increased (Kim *et al.*, 2002; Uccelletti *et al.*, 2002; Jiang *et al.*, 2007; Tanaka *et al.*, 2012).

The hemiascomycetes yeast *Yarrowia lipolytica* has gained a strong industrial interest because of its ability to grow on n-paraffins and produce high amounts of organic acid (Tsugawa *et al.*, 1969). More recently, *Y. lipolytica* has been raised as a potential host for the production of heterologous proteins due to its capacity to secrete high levels of large proteins such as alkaline extracellular protease, lipase, and RNase (Madzak *et al.*, 2004). This yeast is admittedly nonpathogenic and several processes based on *Y. lipolytica* were classified as GRAS. In *Y. lipolytica*, only a few cell wall proteins have been characterized, including YlCwp1p (Jaafar and Zueco, 2004), YlYwp1p (Ramon *et al.*, 1999), and YlPir1p (Jaafar *et al.*, 2003). Displays developed to date with *Y. lipolytica* as a host have mainly employed the glycosylphosphatidylinositol (GPI) domains of YlCwp1p and YlYwp1p, which are fused at the C-terminus of the target protein (Tanaka *et al.*, 2012).

α-1,2-Mannosidases can specifically remove a single mannose residue in α-1,2-linkage from an oligomannosyl glycan substrate. Due to its high specificity towards the α-1,2-mannosidic linkage, these enzymes are used as effective and powerful tools for structural or quantitative analysis of oligosaccharides (Kimura and Kitahara, 2000). Moreover, the introduction of a heterologous α-1,2-mannosidase into the secretion pathway of several yeast species has been reported as an efficient strategy to endow yeast cells the ability to trim highly mannosylated *N*-glycans to homogeneous human-like high mannose-type glycans, $Man_5GlcNAc_2$ or $Man_3GlcNAc_2$, in glycoengineered yeast strains (Chiba *et al.*, 1998; Choi *et al.*, 2003; Oh *et al.*, 2008). Here, we report

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Table 1. List of *Y. lipolytica* strains used in this study

Strain	Description	References
SMS397A	*MATA ade1 ura3 xpr2*	Park *et al.* (1997)
IL3	*MATA ade1 ura3 xpr2 leu2::tc*	Bal *et al.* (2013)
IL3/R	IL3 [pINATX2-R]	This study
IL3/LM	IL3 [pINATX2-LM]	This study
IL3/XM	IL3 [pINAX2-XM]	This study
IL3/AM	IL3 [pINAX2-AM]	This study
IL3/RM	IL3 [pINATX2-RM]	This study
IL3/LMC	IL3 [pINATX2-LMC]	This study
IL3/LMY	IL3 [pINATX2-LMY]	This study
IL3/2x LMC	IL3 [pINATX2-LMC, pIMR53-LMC]	This study
IL3/2x LMY	IL3 [pINATX2-LMY, pIMR53-LMY]	This study
IL3/LMC+LMY	IL3 [pINATX2-LMC, pIMR53-LMY]	This study
Δ*och1*Δ*mpo1*	*MATA ade1 ura3 xpr2 och1::tc mpo1::tc*	Park *et al.* (2011)
Δ*och1*Δ*mpo1*/LM	Δ*och1*Δ*mpo1* [pIMR53-LM]	This study
Δ*och1*Δ*mpo1*/LMC	Δ*och1*Δ*mpo1* [pIMR53-LMC]	This study
Δ*och1*Δ*mpo1*/LMY	Δ*och1*Δ*mpo1* [pIMR53-LMY]	This study

the expression of *Aspergillus saitoi* α-1,2-mannosidase (EC 3.2.1.113) on the cell surface of *Y. lipolytica* using GPI anchoring motifs derived from two cell wall proteins, YlCwp1p and YlYwp1p. We show that the recombinant *Y. lipolytica* strains displaying α-1,2-mannosidase on their cell surface were able to convert $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ efficiently, demonstrating its potential use in glycoengineering *in vitro* or *in vivo*.

Materials and Methods

Strains, plasmids, and growth media

The *Y. lipolytica* strains and plasmids used in this study are listed in Tables 1 and 2. *Y. lipolytica* cells were grown in YPD (1% yeast extract, 2% bacto peptone, 2% glucose) or synthetic complete medium (SC; 0.67% yeast nitrogen base without amino acids, 2% glucose, 1× drop-out amino acid mixture) at 28°C and was transformed using a one-step method (Chen *et al.*, 1997). For selection of Leu^+ or Ura^+ transformants, synthetic complete medium without leucine (SC-LEU) or uracil (SC-URA) was used.

Construction of expression vectors for secretory and surface display of *A. saitoi* msdS

Plasmids for secretory expression of *A. saitoi* α-1,2-mannosidase (msdS) were constructed as follows: First, using pDMMOX-aaMSF-TIP1 (Cheon *et al.*, 2009) as a template, the DNA fragment encoding the mature form of *A. saitoi* msdS fused with a secretion signal sequence (ss) of *A. niger* α-amylase tagged with cMyc at its N-terminus and fused with a FLAG tag at the C-terminus, respectively (*A. niger* α-amylase ss-cMyc-msdS-FLAG) was amplified by polymerase chain reaction (PCR) with the primers MsdS-fw and MsdS-rv

Table 2. List of plasmids used in this study

Plasmid	Description	References
pINAScTRP1	p*TEF1-ScTRP1*-t*XPR2 YlLEU2 ARS68*	Cheon *et al.* (2003)
pDMMOX-aaMSF-TIP1	p*HpMOX- A. niger* α-amylase ss-*cMyc-msdS*-FLAG	Cheon *et al.* (2009)
pINATX2-msdS	p*TEF1-A. niger* α-amylase ss-*cMyc-msdS*-FLAG-t*XPR2 YlLEU2 ARS68*	This study
pINATX2-L	p*TEF1-LIP2* prepro-t*XPR2* in pINATX2	This study
pINATX2-X	p*TEF1-XPR2* prepro-t*XPR2* in pINATX2	This study
pINATX2-A	p*TEF1-A. niger* α-amylase ss-t*XPR2* in pINATX2	This study
pINATX2-R	p*TEF1*-rice α-amylase ss t*XPR2* in pINATX2	This study
pINATX2-LM	p*TEF1-LIP2* prepro *msdS*-FLAG-t*XPR2* in pINATX2	This study
pINATX2-XM	p*TEF1-XPR2* prepro-*msdS*-FLAG-t*XPR2* in pINATX2	This study
pINATX2-AM	p*TEF1-A. niger* α-amylase ss-*msdS*-FLAG-t*XPR2* in pINATX2	This study
pINATX2-RM	p*TEF1*-rice α-amylase ss-*msdS*-FLAG-t*XPR2* in pINATX2	This study
pINATX2-LMC	p*TEF1-LIP2* prepro ss-*msdS*-FLAG-*YlCWP1*-t*XPR2* in pINATX2	This study
pINATX2-LMY	p*TEF1-LIP2* prepro ss-*msdS*-FLAG-*YlYWP1*-t*XPR2* in pINATX2	This study
pIMR53_AUX	p*XPR2*-t*XPR2 YlURA3 ARS18*	Yang *et al.* (2001)
pIα-ACwp1p	p*XPR2*-rice α-amylase-cMyc-*YlCWP1*-t*XPR2* in pIMR53_AUX	Lee (2005)
pIMR53-LM	p*TEF1-LIP2* prepro *msdS*-FLAG-t*XPR2* in pIMR53	This study
pIMR53-LMC	p*TEF1-LIP2* prepro-*msdS*-FLAG-*YlCWP1*-t*XPR2* in pIMR53	This study
pIMR53-LMY	p*TEF1-LIP2* prepro-*msdS*-FLAG-*YlYWP1*-t*XPR2* in pIMR53	This study

Table 3. List of primers used in this study

Primer	Sequence (5′ to 3′)	Specificity
LM-fw	agaggatccatgaagctttccaccatcctc	Amplification of *YlLIP2* ss
LM-rv	tcttctagatcgcttctggagaactgcg	Amplification of *YlLIP2* ss
XM-fw	agaggatccatgaagctcgctaccgcc	Amplification of *YlXPR2* ss
XM-rv	tcttctagatcgcttggcattagaagaagc	Amplification of *YlXPR2* ss
AM-fw	tcttctagaggccaaagcaggtgccgc	Amplification of *A. niger* α-amylase ss
AM-rv	tcttctagaggccaaagcaggtgccgcga	Amplification of *A. niger* α-amylase ss
RM-fw	agaggatccatgcaggtgctgaacacca	Amplification of rice α-amylase ss
RM-rv	tcttctagacccggctgtcaagttgga	Amplification of rice α-amylase ss
MsdS-fw	agaggatccatggtcgcgtggtggtct	Amplification of *A. niger* α-amylase ss-*msdS*
MsdS-rv	agtgatatcgcggccgcttagtcgaccttatcgtcgtcatc	Amplification of *A. niger* α-amylase ss-*msdS*
CWP1-fw	tcaccctcgagaagggctac	Amplification of the *YlCWP1* gene fragment
CWP1-rv	actagcggccgcttaaatgaggagagcggcg	Amplification of the *YlCWP1* gene fragment
YWP1-fw	agtggtcgacgttactgacactgactgcgacg	Amplification of the *YlYWP1* gene fragment
YWP1-rv	actagcggccgctcattgatcaaaaaacttagagaagag	Amplification of the *YlYWP1* gene fragment

The PCR fragment was treated with *Bam*HI and *Sph*I/Klenow and ligated with the *Bam*HI/*Eco*RV-digested pINAScTRP1 (Cheon *et al.*, 2003), resulting in pINATX2-msdS. The DNA fragments encoding the *Y. lipolytica* LIP2 prepro (33 aa) and XPR2 prepro (149 aa) regions were amplified by PCR from *Y. lipolytica* genomic DNA using the primer pairs LM-fw and LM-rv for LIP2 prepro and XM-fw and XM-Rv for *XPR2* prepro (Table 3). The DNA fragments encoding *A. niger* α-amylase ss (20 aa) and rice α-amylase ss (31 aa) were amplified from pDMMOX-aaMSF-TIP1 and pIα-ACwp1p (Lee, 2005), respectively, by PCR using the primer pairs AM-fw and AM-rv for *A. niger* α-amylase ss and RM-fw and RM-Rv for rice α-amylase ss (Table 3). The PCR products of signal sequences were digested with *Bam*HI and *Xba*I, and subcloned into the corresponding site in pINATX2-msdS to replace the DNA fragment encoding the *A. niger* α-amylase ss-cMyc-msdS. The resulting plasmids were named pINATX2-L, pINATX2-X, pINATX2-A, and pINATX2-R, respectively. Next, the *Xba*I-digested DNA fragment encoding the mature msdS, obtained from pINATX2-msdS, was reinserted in frame into the *Xba*I site of pINATX2-L, pINATX2-X, pINATX2-R, and pINATX2-A, generating pINATX2-LM, pINATX2-XM, and pINATX2-AM, and pINATX2-RM, respectively (Fig. 1A). To construct the msdS expression vector containing the *URA3* selection marker, the *Eco*RI/*Not*I-digested p*TEF1-LIP2prepro-msdS*-FLAG fragment from pINATX2-LM was subcloned into the corresponding site of pIMR53_AUX, resulting in the expression vector pIMR53-LM (Fig. 1A).

Plasmids for cell surface display of *A. saitoi* α-1,2-mannosidase were constructed as follows: The DNA fragments encoding the partial C-terminal part of YlCwp1 (381 base pairs, bp) and YlYwp1 (726 bp) proteins were amplified from the genomic DNA of *Y. lipolytica* by PCR, digested with *Sal*I/*Not*I, and then ligated with the *Sal*I/*Not*I-digested pINATX2-LM, generating pINATX2-LMC and pINATX2-LMY, respectively (Fig. 1B). On the other hand, the PCR fragments of *YlCWP1* and *YlYWP1* were inserted into the *Sal*I/*Not*I sties of pIMR53-LM, generating pIMR53-LMC and pIMR53-LMY, respectively (Fig. 1B).

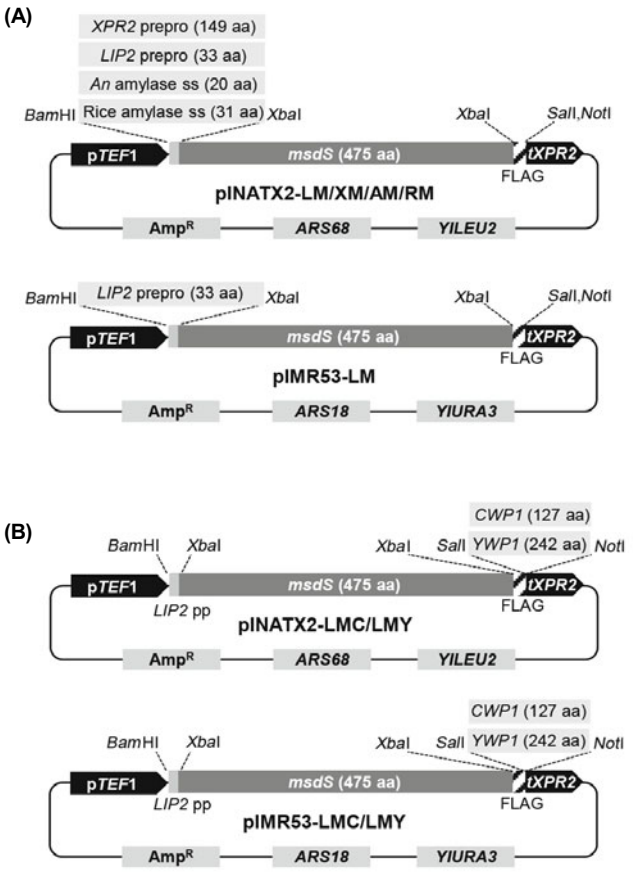

Fig. 1. Schematic representation of expression vectors for secretion and surface display of *A. saitoi* msdS. (A) Vectors for secretory expression of msdS based on pINATX2 (upper panel) and pIMR53 (lower panel). p*TEF1*, the promoter of *Y. lipolytica TEF1*; *XPR2* prepro, secretion signal from *Y. lipolytica* AEP; *LIP2* prepro, secretion signal from *Y. lipolytica* extracellular lipase; *An* amylase ss, secretion signal from *A. niger* α-amylase; Rice amylase ss, secretion signal from rice α-amylase; t*XPR2,* the terminator of *Y. lipolytica* AEP; *YlLEU2* and *YlURA3*, selective markers of *Y. lipolytica*; *ARS18* and *ARS68*, autonomous replication sequences of *Y. lipolytica*; Amp^R, the *bla* gene; FLAG, a single copy of FLAG tag. (B) Vectors for surface display of msdS on the cell wall of *Y. lipolytica* based on pINATX2 (upper panel) and pIMR53 (lower panel), respectively. The GPI-anchor motifs of *Y. lipolytica* Cwp1 or Ywp1 proteins were fused at the C-terminus of Lip2 prepro-msdS.

Protein sample preparation and western blot analysis

The transformed yeast cells were inoculated in YPD medium and aerobically cultivated at 28°C for 48 h. Yeast cells were collected by centrifugation (3,000×*g*, 10 min), and washed twice with chilled distilled water. For preparation of soluble cytosolic fraction, the yeast cells were suspended in TNE buffer (0.5 M EDTA, 5 M NaCl, 1 M Tris-HCl, pH 7.5) with a small volume of 1 mM PMSF, protein inhibitor cocktail (P8215, Sigma, USA), and broken by vortexing with glass beads. After centrifugation (16,000×*g*, 20 min, 4°C), the supernatant was isolated as soluble cytosolic fraction. For preparation of the cell wall fraction, the cell pellet was washed with chilled distilled water, and was then resuspended in SDS/DTT extract solution (50 mM Tris-HCl; pH 8.0, 0.1 M EDTA, 2% SDS, 10 mM DTT). Finally, the cytosolic fraction, SDS/DTT extract, and culture supernatant fractions were boiled for 10 min in SDS-PAGE sample loading buffer (0.25% bromophenol blue, 0.5 M DTT, 50% glycerol, 10% SDS, 0.25 M Tris-HCl, pH 6.8) and separated by SDS-PAGE. The gels were stained with Coomassie brilliant blue R-250 or analyzed using western blot using an anti-FLAG M2 monoclonal antibody at a dilution of 1:5000 dilution (Sigma) and detected with the AP Conjugate Substrate Kit (Bio-Rad, USA).

N-glycan structural analysis of cell wall glycoproteins by HPLC

The cell wall mannoproteins were prepared using an earlier protocol (Park *et al.*, 2011). In brief, yeast cells were resuspended in 100 mM citrate buffer (1 M sodium citrate, 1 M citric acid, pH 7.0) in a ratio of 1:1 (w/v). The mixture was heated at 121°C for 90 min and the supernatant was isolated by centrifugation (16,000×*g*, 20 min, 4°C). The total cell wall mannoproteins were precipitated from the supernatant using 3 volumes of 100% cold ethanol and dissolved in HPLC water for glycan analysis. The obtained total cell wall proteins were treated with 500 U of PNGase F (peptide: *N*-glycosidase F, NEB, UK) for 16 h at 37°C, and the released *N*-linked glycans were purified using a Carbograph Extract-Clean column (Alltech, USA). The purified glycans were then labeled with 2-aminobenzoic acid (AA) and were further purified using SPE (Solid Phase Extraction, Agilent Technologies, USA). The AA-labeled oligosaccharide products were analyzed by Shodex Asahipak NH2P-50 4E (4.6 mm × 250 mm) (Shoko, Japan) at a flow rate of 1.0 ml/min by gradient chromatography using solvents A (97% acetonitrile, 2% acetic acid and 1% tetrahydrofuran) and B (91% HPLC water, 5% acetic acid, 3% triethylamine and 1% tetrahydrofuran). After sample injection, the proportion of

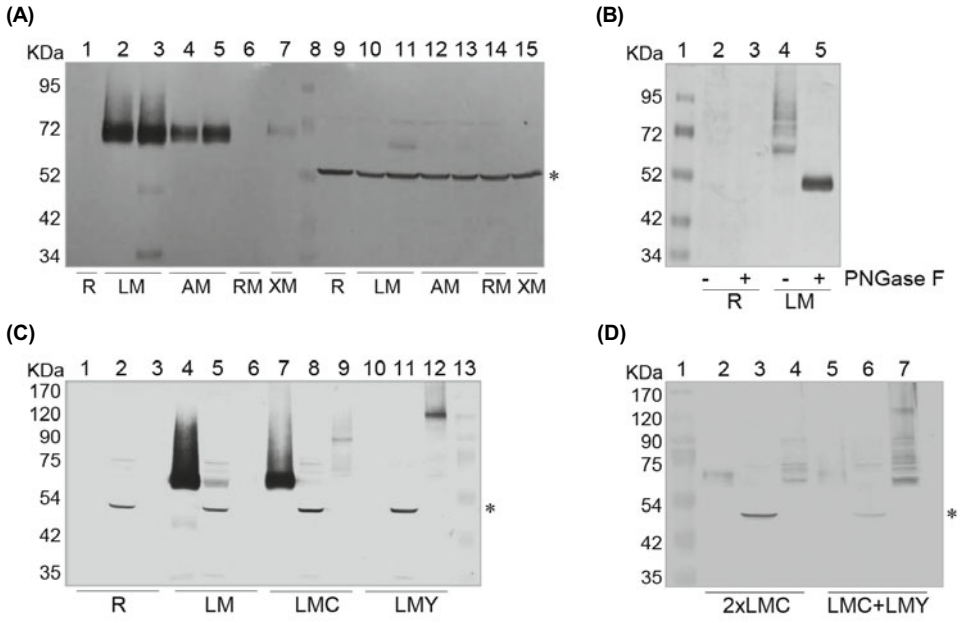

Fig. 2. Secretion and surface display of glycosylated msdS in *Y. lipolytica*. (A) Expression analysis of the secretory msdS proteins using various signal sequences (ss): *LIP2* prepro (lanes 2, 3, 10 and 11), *XPR2* prepro (lanes 7 and 15), rice α-amylase ss (lanes 6 and 14), and *A. niger* α-amylase ss (lanes 4, 5, 12 and 13). Western blot analysis was carried out using anti-FLAG antibody with culture supernatants (lanes 1 to 7), and total cell extracts (lanes 9 to 15). (B) Glycosylation pattern analysis of the secretory msdS. The culture supernatant of *Y. lipolytica* wild-type strains harboring the control vector pINATX2-R (strain IL3/R) and the msdS expression vector pINATX2-LM (strain IL3/LM), respectively, were incubated without (lanes 2 and 4) and with PNGase F (lanes 3 and 5). Lanes 2 and 3, samples from the strain IL3/R; lanes 4 and 5, samples from the strain IL3/LM. (C) Expression analysis of msdS-GPI fusion construct from a single expression vector containing *YlLEU2* as selection marker. Western blot using anti-FLAG antibody was carried out on the culture supernatant (lanes 1, 4, 7, and 10), soluble cell extract (lanes 2, 5, 8, and 11) and SDS/DTT-extracted cell wall protein fractions (lanes 3, 6, 9, and 12) from *Y. lipolytica* transformants. Lanes 1, 2 and 3, strain IL3/R; lanes 4, 5 and 6, strain IL3/LM; lanes 7, 8 and 9, strain IL3/LMC; lanes 10, 11 and 12, strain IL3/LMY. (D) Analysis of msdS-GPI expression from two vectors containing *YlLEU2* and *YlURA3* as selection markers, respectively. The culture supernatant (lanes 2 and 5), soluble cell extract (lanes 3 and 6), and SDS/DTT-extracted cell wall protein fractions (lanes 4 and 7) were prepared from strains IL3/2xLMC (lanes 1, 2, and 3) and IL3/LMC+LMY, respectively. A non-specific band is indicated with an asterisk. Lane 8 in A, lane 1 in B, lane 13 in (C) and lane 1 in (D) contain protein molecular weight markers [Fermentas (USA) and ELPIS-Biotech (Korea)].

solvent B was increased linearly for 90 min from 30% to 70%. AA-oligosaccharides were detected by fluorescence (lex = 320 nm and lem = 400 nm) with a Waters 2475 fluorescence detector (Waters, USA). The glycan structures were determined by comparing the retention times with AA-labeled standards.

In vitro α-1, 2-mannosidase activity analysis

Pre-cultured yeast cells in SC-LEU medium were cultured in YPD for 48 h. About 10^6 yeast cells were resuspended in 0.5 ml reaction buffer (20 mM ammonium acetate buffer, pH 5.4) containing 2-AA-labeled $Man_8GlcNAc_2$, and the reaction was carried out at 37°C overnight and centrifuged at 4,000×*g* for 10 min. The supernatant was purified by an Amicon column (YM30, Millipore, USA) and subjected to NP-HPLC (normal-phase high-pressure liquid chromatography) analysis.

Surface expression analysis of msdS by flow cytometry

Flow cytometry was performed to confirm the surface display of msdS. The transformants were harvested by centrifugation and rinsed with TBS buffer (10 mM Tris-HCl; pH 8.0, 0.15% NaCl). Approximately 10^7 cells in 250 μl TBS buffer were incubated with anti-FLAG monoclonal antibody (1:100) at 4°C for 12 h. After the cells were rinsed with TBS buffer twice, they were labeled with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (1:100) at 4°C for 1 h. The labeled yeast cells were washed with TBST buffer (10 mM Tris-HCl; pH 8.0, 0.15% NaCl, 0.05% Tween 20) twice, and then sorted by BD FACSCalibur™ (BD Bioscience, USA). The emission of FITC signal was detected in the FL1 channel.

Results and Discussion

Expression of secretory *A. saitoi* msdS in *Y. lipolytica*

A secretion signal is a prerequisite for cell surface expression of a protein to efficiently direct it towards the secretion pathway of the cell. For optimizing secretion of *A. saitoi* α-1,2-mannosidase in *Y. lipolytica*, four different signal sequences, including the *Y. lipolytica* endogenous prepro sequences of an alkaline extracellular protease (AEP) encoded by *XPR2* and a lipase encoded by *LIP2,* and heterologous signal sequences from *A. niger* and rice α-amylases, were analyzed for their secretion efficiency. The secretory expression vectors were constructed using the plasmid pINATX2 as a backbone vector that contains the *TEF1* (translation elongation factor 1α) promoter, the *XPR2* terminator, *YlLUE2*, and *ARS68* (autonomously replicating sequence 68). The four signal sequences were fused with the *A. saitoi msdS* gene encoding a mature form of α-1,2-mannosidase with 475 amino acid residues without the signal sequence region (Fig. 1A). The secreted *A. saitoi* msdS protein, tagged with the C-terminal FLAG epitope, was detected with the anti-FLAG antibody in western blot analysis. It was shown that the most intense msdS protein signal was detected in the culture supernatant of the fusion construct using the *LIP2* prepro region (Fig. 2A). The secreted *A. saitoi* msdS was detected as a smear, much larger than the predicted size of an unglycosylated mature form with a molecular weight of ~53 kDa. The result suggested that the recombinant *A. saitoi*

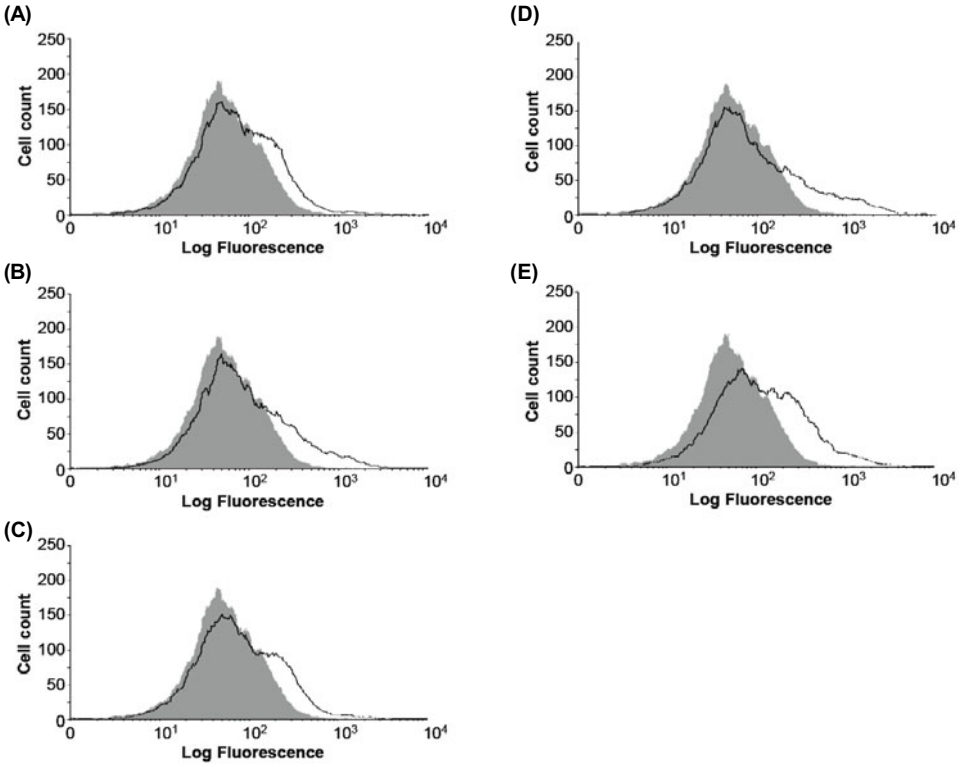

Fig. 3. Flow cytometry analyses of *Y. lipolytica* cells displaying msdS-GPI anchor fusion proteins. Flow cytometry analysis was performed by staining *Y. lipolytica* strains expressing msdS-GPI fusion constructs, (A) IL3/LMC, (B) IL3/LMY, (C) IL3/2x LMC, (D) IL3/2x LMY, and (E) IL3/LMC+LMY, with anti-FLAG antibody and anti-mouse IgG antibody conjugated with FITC. The black peak represents the histogram from *Y. lipolytica* strain containing a negative control vector (IL3/R) as a reference.

msdS was secreted in a heterogeneously hyper-mannosylated form in *Y. lipolytica*.

To investigate glycosylation in the recombinant *A. saitoi* msdS, we analyzed the band pattern of secreted proteins by SDS-PAGE after PNGase F treatment, which specifically catalyzes the cleavage of *N*-linked oligosaccharides between the innermost GlcNAc of oligosaccharides and asparagine residues of glycoproteins. After treatment with PNGase F, the smear obtained earlier (Fig. 2B, lane 4) was compressed into a single band around ~50 kDa, a little smaller than the expected size of 53 kDa (Fig. 2B, lane 5). We had previously observed that the *A. saitoi* msdS secreted in *H. polymorpha* was also detected at the position of ~50 kDa after PNGase F treatment (Kim *et al.*, 2006). Considering that the molecular weight of completely glycosylated msdS with 8 putative glycosylation sites is about 72 kDa, these results indicated that the recombinant *A. saitoi* α-1,2-mannosidase expressed in yeast *Y. lipolytica* appeared to have all the expected *N*-glycans with hyper-mannosylation.

Targeted expression of *A. saitoi* msdS at the cell surface of *Y. lipolytica*

The cell surface display of enzymes has gained interest because of simpler purification steps and the possibility of recycling the enzyme in industrial process (Yuzbasheva *et al.*, 2011). To display *A. saitoi* msdS on the cell surface of *Y. lipolytica*, the DNA fragments encoding the GPI-anchor domains of YlCwp1p (127 amino acids) and YlYwp1p (242 amino acids), respectively, were fused at the C-terminal of the *LIP2* prepro-*msdS* gene. Two expression vectors, pINATX2-LMC and pINATX2-LMY, were introduced into *Y. lipolytica* IL3 wild-type strain for surface display of msdS, resulting in the transformants IL3/LMC and IL3/LMY, respectively. The expression and localization of the msdS-CWP1 and msdS-YWP1 fusion proteins were analyzed with the fractionated protein samples obtained from the IL3/LMC and IL3/LMY strains. The IL3/LM strain harboring the secretory expression vector pINATX2-LM was used as control.

In western blot analysis with an anti-FLAG monoclonal antibody, the bands corresponding to msdS-CWP1 and msdS-YWP1 fusion proteins were detected only in the SDS/DTT-extracted cell wall protein samples of IL3/LMC and IL3/LMY transformants, not in the soluble cytosol and culture supernatant fractions (Fig. 2C, lanes 7 to 12). For the *Y. lipolytica* IL3/LM transformant used as a control strain, most of msdS was secreted into the supernatant without accumulation in the SDS/DTT extract fraction (Fig. 2C, lanes 4 to 6). The expected molecular weight of msdS-CWP1 fusion protein with 8 *N*-linked glycans on msdS and 8 *O*-linked glycans on the GPI domain of YlCwp1p is about 92 kDa, and that of msdS-YWP1 with 8 *N*-linked glycans on msdS and 28 *O*-linked glycans on YlYwp1p is about 126 kDa, respectively. These results indicate that the msdS proteins fused with GPI motifs successfully displayed *N*- and *O*-glycosylations on the cell surface of yeast *Y. lipolytica*. It is worth noting that the msdS-YWP1 fusion construct resulted in recombinant fusion protein localized mostly in the cell wall fraction without significant secretion. On the other hand, with the msdS-CWP1 fusion construct, a substantial amount of msdS protein lacking the fused CWP1 was observed in the culture supernatant fraction (Fig. 2C, lane 7). Such a high level of secretion of msdS lacking CWP1 fusion could be explained by proteolytic cleavage at the junction between msdS and the GPI domain of YlCwp1p during protein secretion or after cell surface anchorage.

Furthermore, we tried to increase the expression level of msdS fusion protein in the cell wall fraction by combinatorial introduction of msdS-CWP1 and/or msdS-YWP1 expression vectors containing two different selective markers and *ARS* sequences (Fig. 2D). The pINATX2 series vectors containing *YlLEU2* as a selection marker are based on *ARS68*, whereas the pIMR53 series vectors containing *YlURA3* are based on *ARS18* (Table 2). It was previously reported that these *ARS* fragments contain *Y. lipolytica* centromeres, so that vectors containing them are mitotically stable but maintained mostly in low copies, about 3 per cell (Fournier *et al.*,

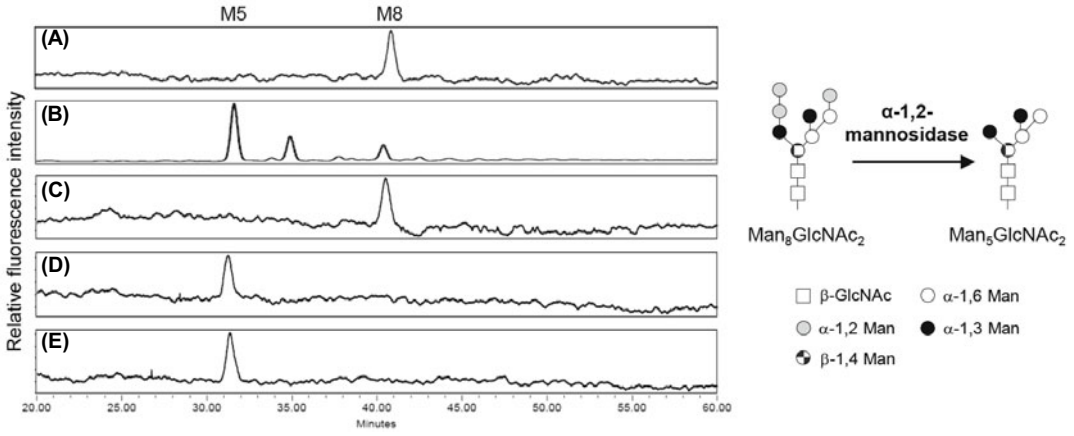

Fig. 4. *In vitro* activity analysis of surface displayed msdS by HPLC. Whole cells of *Y. lipolytica* transformants displaying msdS on the cell surface were used as enzyme source, and $Man_8GlcNAc_2$-AA as a substrate. After incubation at 37°C overnight, the reaction products were analyzed by HPLC. (A) Negative control reaction with buffer only. (B) Positive control reaction with the purified commercial *A. saitoi* α-1,2-mannosidase (Prozyme). (C) Negative control reaction with *Y. lipolytica* cells harboring a negative control vector (IL3/R). (D) Reaction with the *Y. lipolytica* strain displaying the msdS-CWP1 fusion construct (IL3/LMC). (E) Reaction with the *Y. lipolytica* strain displaying msdS-YWP1 fusion construct (IL3/LMY).

1993). Co-transformation with two vectors would be expected to increase the copy number of the msdS-fusion expression unit, resulting in increased expression of surface-displayed msdS in *Y. lipolytica*.

Flow cytometry analysis of surface-displayed *A. saitoi* msdS in *Y. lipolytica*

The expression of msdS-CWP1 and msdS-YWP1 fusion proteins on the surface of *Y. lipolytica* cells was further confirmed by flow cytometry analysis of cells labeled with immunofluorescent FITC. Increased fluorescence signals were obtained from strains expressing msdS fused with the GPI motifs of YlCwp1p or YlYwp1p compared to autofluorescence of the reference strain harboring a negative control vector (pINATX2-R) (Figs. 3A and 3B). Moreover, an improved shift of the fluorescence histogram was clearly detected in the IL3/LMC+LMY strain harboring the two different expression vectors, pINATX2-LMC and pIMR53-LMY, exploiting the GPI motifs of YlCwp1p and YlYwp1p, respectively (Fig. 3E). Thus, these results further supported the fact that the msdS proteins fused with GPI-anchored proteins were efficiently displayed on the surface of the *Y. lipolytica* and further enhancement of display expression could be achieved by an increase in copy number.

In general, the degree of shift of the fluorescence histogram reflects the expression efficiency of surface-expressed target proteins. Thus, the pattern of fluorescence shift in Fig. 3 indicates that the expression levels of msdS displayed on the surface of *Y. lipolytica* appear overly low. Alternatively, it can be interpreted that the accessibility to the FLAG epitope might be partially limited due to its location in the middle of msdS-CWP1 and msdS-YWP1 fusion constructs, resulting in low fluorescence intensity.

In vitro activity analysis of surface-displayed *A. saitoi* msdS

To confirm the functional expression of recombinant msdS displayed on the cell surface of *Y. lipolytica*, the α-1,2-mannosidase activity of whole yeast cells displaying msdS were assayed using AA-labeled $Man_8GlcNAc_2$ glycan as substrate. Whole cells of *Y. lipolytica* IL3/LMC and IL3/LMY strains expressing msdS-CWP1 or msdS-YWP1 fusion protein on their cell surface were harvested and incubated with AA-labeled $Man_8GlcNAc_2$ at 37°C overnight. After purification using an Amicon column, the reaction mixture was subjected to HPLC analysis. Compared with negative control (*Y. lipolytica* cells transformed with the negative control vector pINATX2-R), both cell surface displaying msdS-CWP1 and msdS-YWP1 fusion proteins led to a clear shift from $Man_8GlcNAc_2$ peak to $Man_5GlcNAc_2$ peak (Fig. 4). These results indicate that the surface displayed *A. saitoi* msdS completely cleaved three α-1,2-mannosidic linkages in the substrate $Man_8GlcNAc_2$, resulting in the final product $Man_5GlcNAc_2$.

Expression of *A. saitoi* msdS in *Y. lipolytica* Δ*och1*Δ*mpo1* mutant strain

In an effort to develop glycoengineered *Y. lipolytica* strains possessing a mannose trimming capacity, the secretory and surface display expression of *A. saitoi* msdS was tried in the *Y. lipolytica* Δ*och1*Δ*mpo1* double mutant strain. The Δ*och1* Δ*mpo1* strain contains deletions of the genes *YlOCH1* and *YlMPO1* that prevent yeast-specific processing of *N*-glycans such as the initiation of synthesis of the outer mannose chain (Song *et al.*, 2007; Park *et al.*, 2011). The surface-displayed msdS expression vectors harboring *YlURA3* as a selection marker, pIMR53-LM, pIMR53-LMC, and pIMR53-LMY, were constructed (Fig. 1) and introduced into the Δ*och1*Δ*mpo1* double mutant strain. In western blot analysis, whereas the secretory msdS was detected as a smear in wild-type strain (Fig. 5A, lane 5), it was detected as a single band in the Δ*och1*Δ*mpo1* double deletion strain (Fig. 5A, lane 9). Expectedly, the Δ*och1*Δ*mpo1* strains expressing the surface-displayed msdS-CWP1 or msdS-YWP1 fusion protein also showed more homogeneous bands as major species in the SDS/DTT extract samples (Fig. 5B, lanes 9 and 12). These

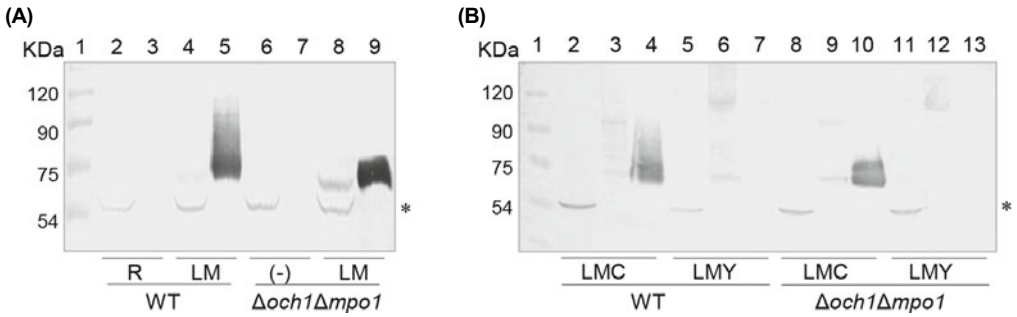

Fig. 5. Expression analysis of msdS-GPI fusion constructs in the glycoengineered *Y. lipolytica* strain. (A) Western blot analysis of secretory expression of msdS in the Δ*och1*Δ*mpo1* strain. Total cell lysate (lanes 2, 4, 6, and 8) and culture supernatant of the wild type and Δ*och1*Δ*mpo1* transformants were analyzed by western blot with an anti-FLAG antibody. Lane information is as follows; wild-type strain IL3/R (lanes 2 and 3); IL3/LM (lanes 4 and 5); Δ*och1*Δ*mpo1* (lanes 6 and 7); and Δ*och1*Δ*mpo1*/LM (lanes 8 and 9). (B) Western blot analysis of msdS surface expression in the Δ*och1*Δ*mpo1* strain. The soluble cytosolic fraction (lanes 2, 5, 8, and 11), the SDS/DTT-extracted protein fraction (lanes 3, 6, 9, and 12), and culture supernatant fraction (lanes 4, 7, 10, and 13) were prepared from the recombinant wild-type IL3 strain and Δ*och1*Δ*mpo1* strains and subjected to western blot analysis with an anti-FLAG antibody. Lanes 2 to 4, samples from wild-type strain IL3/LMC; lanes 5 to 7, samples from IL3/LMY; lanes 8 to 10, samples from Δ*och1*Δ*mpo1*/LMC; lanes 11 to 13, samples from Δ*och1*Δ*mpo1*/LMY. A non-specific band is labeled with an asterisk. Lane 1 in both (A) and (B) contain the protein molecular weight marker (Fermentas).

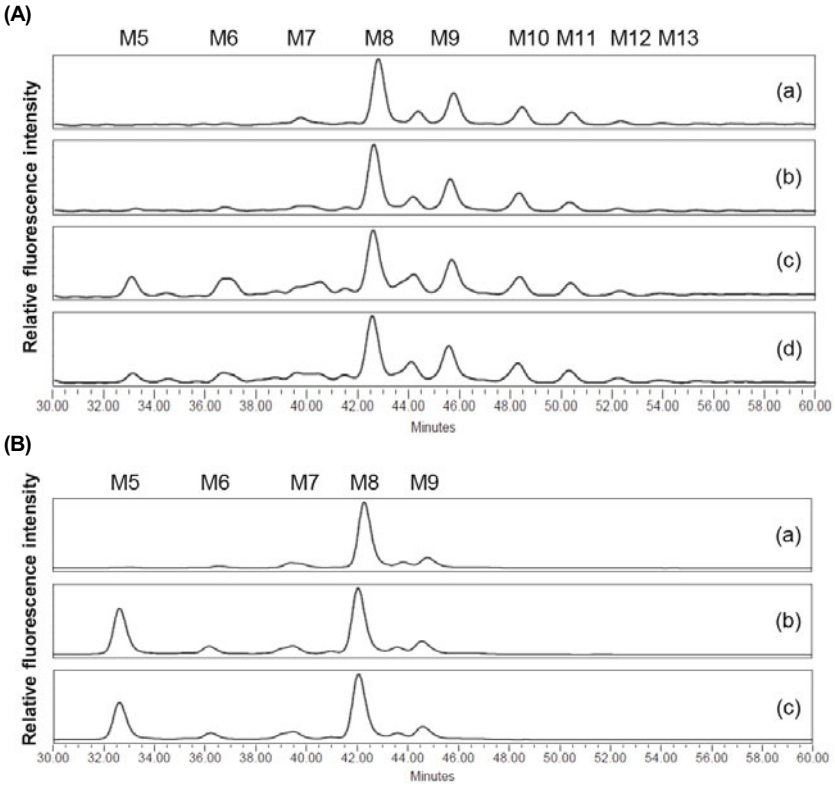

Fig. 6. HPLC analysis of *N*-glycan profiles of *Y. lipolytica* displaying *A. saitoi* msdS. (A) *N*-glycan profiles of recombinant wild-type IL3 strains harboring a negative control vector (a, strain IL3/R), an expression vector for secretory msdS (b, IL3/LM), a surface display vector for msdS-CWP1 (c, IL3/LMC), and a surface display vector for msdS-YWP1 (d, IL3/LMY). (B) The *N*-glycan profile of recombinant Δ*och1*Δ*mpo1* mutant strains harboring a negative control vector (a), Δ*och1*Δ*mpo1*/LMC with surface displayed msdS-CWP1 (b), and Δ*och1*Δ*mpo1*/LMY with msdS-YWP1 (c). *N*-linked oligosaccharides from total cell wall proteins were labeled with AA and analyzed by NP-HPLC. The peaks M5 to M13 represent $Man_{5-13}GlcNAc_2$ *N*-glycans. Y-axis represents relative fluorescence intensity in NP-HPLC analysis. The differences in the scale of fluorescence intensity in the Y-axis reflect concentrations of the cell wall glycans used for the analysis.

data clearly show that the Δ*och1*Δ*mpo1* double mutant strain produced secretory and surface-displayed msdS proteins in homogeneously mannosylated glycoforms due to the lack of yeast specific hypermannosylation and phosphorylation.

In vivo activity analysis of surface-displayed *A. saitoi* msdS

In order to test *in vivo* activity of *A. saitoi* msdS during secretion process, we analyzed the *N*-glycan profiles of cell wall proteins prepared from strains expressing secretory or cell surface msdS. The *N*-glycan profiles of cell wall mannoproteins showed that the wild-type strains without msdS expression or with secretory expression of msdS produced *N*-glycans larger than $Man_{8-13}GlcNAc_2$ as the major species (Fig. 6A, a and b). In contrast, the shorter $Man_{5-10}GlcNAc_2$ glycans were detected in the wild-type strains expressing surface-displayed msdS (Fig. 6A, c and d), indicating that some portion of *N*-glycans are trimmed by α-1,2-mannosidase activity of msdS displayed on the cell surface.

In the Δ*och1*Δ*mpo1* double mutant strain background, the core form $Man_8GlcNAc_2$ was detected as a major species of *N*-glycans (Fig. 6B, a). In contrast, a substantial portion of $Man_8GlcNAc_2$ was converted to $Man_5GlcNAc_2$ in Δ*och1*Δ*mpo1* double mutant transformants expressing surface-displayed *A. saitoi* msdS (Fig. 6B, b and c). These results show the glycoengineered *Y. lipolytica* expressing surface-displayed α-1,2-mannosidase would be useful not only for *in vitro* trimming but also for *in vivo* trimming of *N*-glycans during the protein secretion process in yeast.

In conclusion, by using GPI anchoring motifs from YlCwp1p and YlYwp1p, we have developed a novel cell surface displayed α-1,2-mannosidase in the yeast *Y. lipolytica*, which is fully active against an oligomannosyl glycan substrate. These results present the potential of using the whole *Y. lipolytica* cell with a recombinant cell surface α-1,2-mannosidase as an immobilized enzyme, which can be re-used many times. In addition, the glycoengineered *Y. lipolytica* with surface α-1,2-mannosidase was shown to be useful as a host for the surface expression of glycoproteins with the human mannose-type $Man_5GlcNAc_2$ glycan.

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